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<p>(54) Title: THERAPEUTIC USE OF CIS-ELEMENT DECOYS IN VIVO</p>		
<p>(57) Abstract</p> <p>The invention provides for the use of oligodeoxynucleotide decoys for the prophylactic or therapeutic treatment of diseases associated with the binding of endogenous transcription factors to genes involved in cell growth, differentiation and signalling or to viral genes. By inhibiting endogenous trans-activating factors from binding transcription regulatory regions, the decoys modulate gene expression and thereby regulating pathological processes including inflammation, intimal hyperplasia, angiogenesis, neoplasia, immune responses and viral infection. The decoys are administered in amounts and under conditions whereby binding of the endogenous transcription factor to the endogenous gene is effectively competitively inhibited without significant host toxicity. The subject compositions comprise the decoy molecules in a context which provides for pharmacokinetics sufficient for effective therapeutic use.</p>		

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## THERAPEUTIC USE OF CIS-ELEMENT DECOYS IN VIVO

### INTRODUCTION

5

#### Technical Field

The field of this invention is therapeutic treatment of disease with double stranded nucleic acids which bind transcription factors.

10 Background

A wide variety of diseases result from the over- or under-expression of one or more genes. Given cells may make insufficient amounts of a protein (e.g. insulin) or too much of a protein, be it a normal protein (e.g. TNF), a mutant protein (e.g. an oncogene), or a non-host protein (e.g. HIV-tat). The ultimate goal of therapeutic intervention in such diseases is a selective modulation of gene expression.

A variety of methods of transcriptional modulation in vitro have been reported including the use of anti-sense nucleic acids capable of binding nascent message, intracellular immunization with dominant negative mutants.

With the broad potential therapeutic applications, massive efforts have been extended by prominent laboratories and clinics around the world to extend these methods in vivo. To date, the transcription factor decoy strategy has never been successfully adopted in vivo.

Relevant Literature

Description of the roles of transcription factors may be found in Nevins, *Science* 258, 424-429 (1992); Dalton, *EMBO J.* 11, 11797 (1992); Yee et al. *ibid.* 6, 2061 (1987), Weintraub et al., *Nature* 358, 259-261 (1992), Pagano et al., *Science* 255, 1144-1147 (1992). Viral coat protein-liposome mediated transfection is described by Kaneda et al., *Science* 243, 375 (1989). Ritzenthaler et al. (1991) *Biochem. J.* 280, 157-162; Ritzenthaler et al (1993) *J. Biol. Chem.* 268, 13625-13631; Bielinska et al., *Science* 16, 997-1000 (1990) and Sullenger et al., *Cell* 63, 601-608 (1990) describe inhibition of transcription with double stranded nucleic acids.

A general discussion concerning the mechanism of restenosis may be found in Libby et al., *Circulation* 86, III-47 (1992) and Clowes et al., *J. Cardiovasc. Pharmacol.* 14, S12-15 (1989).

SUMMARY OF THE INVENTION

The invention provides for the therapeutic treatment of diseases associated with the binding of endogenous transcription factors to genes involved in cell growth, differentiation and signalling or to viral genes. Methods and compositions are provided for blocking the capacity of endogenous trans-activating factors to modulate gene expression and thereby regulating pathological processes including inflammation, intimal hyperplasia, angiogenesis, neoplasia, immune responses and viral infection.

The methods comprise administering to a patient double stranded nucleic acid "decoys" in a form such that the decoys are capable of entering target cells of the patient and specifically binding an endogenous transcription factor, thereby competitively inhibiting the transcription factor from binding to an endogenous gene.

The decoys are administered in amounts and under conditions whereby binding of the endogenous transcription factor to the endogenous gene is effectively competitively inhibited without significant host toxicity. Depending on the transcription factor, the methods can effect up- or down-regulation of gene expression. The subject compositions comprise the decoy molecules in a context which provides for pharmacokinetics sufficient for effective therapeutic use.

### DESCRIPTION OF SPECIFIC EMBODIMENTS

Methods and compositions are provided for modulating gene expression in vivo. The methods involve administering a composition to a patient so as to introduce into a target cell molecular decoys comprising double-stranded nucleic acid, usually DNA, to which transcription factors endogenous to the target cell bind. Various methods are employed for in vivo administration of the decoys such that sufficient decoys enter into the target cells to competitively inhibit transcription factor binding to an endogenous gene regulatory region.

The compositions which are employed comprise "decoys": double-stranded nucleic acid molecules with high binding affinity for the targeted transcription factors. The targeted transcription factors are endogenous, sequence-specific double-stranded DNA binding proteins which modulate (increase or decrease) the rate of transcription of one or more specific genes in the target cell. Essentially any such transcription factor (henceforth, "transcription factor") can be targeted so long as a specific decoy capable of competitively inhibiting binding to the endogenous gene can be identified. Numerous transcription factors and their binding sequences are known in the art as are methods for identifying such complements, see e.g. Wang and Reed (1993) *Nature* 364, 121 and Wilson et al. (1991) *Science* 252, 1296. As used herein, endogenous means that the gene or transcription factor is present in the target cell at the time the decoy is introduced.

The transcription factors will, for the most part and depending on the clinical indication, regulate the transcription of genes associated with cell growth, differentiation and signalling or viral genes resident in the target cell. Examples include genes necessary for mitosis, particularly going from G<sub>0</sub> to S, such as proteins associated with check points in the proliferative cycle, cyclins, cyclin dependent kinases, proteins associated with complexes, where the cyclin or cdk is part of the complex, Rosenblatt et al., *Proc. Natl. Acad. Sci.* 89, 2824 (1992) and Pagano et al., *Science* 255, 1144 (1992). Often such genes or the transcription factors themselves will be oncogene products or cellular counterparts, e.g. fos, jun, myc, etc. Other examples include genes encoding secreted proteins and peptides such as hormones e.g. growth factors, cytokines, e.g. interleukins, clotting factors, etc. Target transcription factors also include host and host-cell resident viral transcription factors which activate viral genes present in infected host cells.

Preferred-target transcription factors are activated (i.e. made available in a form capable of binding DNA) in a limited number of specifically activated cells. For example, a stimulus such as a wound, allergen, infection, etc may activate a metabolic pathway that is triggered by the transient availability of one or more transcription factors. Such transcription factors may be made available by a variety of mechanisms such as release from sequestering agents or inhibitors (e.g. NFκB bound to IκB), activation by enzymes such as kinases, translation of sequestered message, etc. Desirably, the target transcription factor(s) will be associated with genes other than genes whose lack of expression results in cytotoxicity. For the most part, it is desirable not to kill the cell; but rather to inhibit or activate specific gene transcription.

Exemplary transcription factors and related cis elements, the cellular processes impacted and therapeutic indication include:

Cis-element Txn Factor	Cellular Process	Therapeutic Application
E2F	cell proliferation	neointimal hyperplasia, neoplasia, glomerulonephritis, angiogenesis, inflammation
AP-1	cell growth, differentiation, growth factor expression	neointimal hyperplasia, cardiac myocyte growth/differentiation
NFκB	cytokine expression, leukocyte adhesion molecule expression, oxidant stress response, cAMP and protein kinase C activation, Ig expression	inflammation, immune response, transplant rejection, ischemia-reperfusion injury, glomerulonephritis
SSRE	response to shear stress: growth factor expression, vasoactive substances, matrix proteins, adhesion molecules.	neointimal hyperplasia, bypass grafts, angiogenesis, collateral formation.
CREB	cAMP response	cAMP activated events



MEF-2	cardiac myocyte differentiation and hypertrophy	cardiac myocyte differentiation and growth.
CARG box	cardiac myocyte differentiation	cardiac myocyte growth and differentiation.
tax	viral replication	HTLV infection
5 VP16	viral replication	Herpes infection
TAR/tat	viral replication	HIV infection
GRE/HRE MRE	glucocorticoid, mineralocorticoid induced events	steroid hormone processes e.g. (breast or prostate cell growth).
10 Heat shock RE	heat shock response	cellular stresses e.g. ischemia, hypoxia
SRE	growth factor responses	cell proliferation/differentiation
AP-2	cAMP and protein kinase response, retinoic acid response	cell proliferation
15 sterol response element	modulation of LDL cholesterol receptor expression	hypercholesterolemia
19 TGFb responsive element	Transforming growth factor beta induced cellular processes	cell growth, differentiation, migration, angiogenesis, intimal hyperplasia, matrix generation, apoptosis.
20		

The length, structure and nucleotide sequence of the decoy will vary depending on the targeted transcription factor, the indication, route of administration, etc. For example, targeted transcription factors frequently bind with different degrees of affinity to a variety of sequences, normally sharing a high degree of homology. Accordingly, one may choose to use a sequence associated with a particular target gene or use a

consensus sequence based on the nucleotide at each site which occurs most frequently in the binding sequences to which the particular transcription factor binds. For example, when targeting a host transcription factor involved in viral transcription, it may be possible to minimize undesirable effects on host transcription is preferably by employing the viral-specific binding sequence. Similarly, where transcription is mediated by a multimeric complex, it is often desirable to target a single transcription factor to minimize effects on non-targeted genes. For example, in the case of Herpes virus transcription, one may target the viral VP16 without concomitant targeting of the promiscuous host Oct protein.

10 In addition to binding affinity, the decoys are also selected for binding specificity. Desirably, the decoys will be highly specific for the target transcription factor(s) such that their effect on nontarget cells and nontargeted metabolic processes of target cells are minimized. Such selection is accomplished in vitro by comparative binding to known transcription factors and nuclear extracts and in culture and in vivo  
15 by assaying nontargeted cell function and effects on nontargeted cell types.

The decoys contain sufficient nucleotide sequence to ensure target transcription factor binding specificity and affinity sufficient for therapeutic effectiveness. For the most part, the target transcription factors will require at least six base pairs, usually at least about eight base pairs for sufficient binding specificity and affinity. Frequently,  
20 providing the decoys with flanking sequences (ranging from about 5 to 50 bp) beside the binding site enhance binding affinity and/or specificity. Accordingly, cis element flanking regions may be present and concatemer oligonucleotides may be constructed with serial repetitions of the binding and/or cis element flanking sequences.

In one embodiment, the decoys are non-replicative oligonucleotides fewer than  
25 100 bp, usually fewer than 50 bp and usually free of coding sequence, being primarily from the non-coding 5' region of a gene. Alternatively, the decoys may comprise a portion of a larger plasmid, including viral vectors, capable of episomal maintenance or constitutive replication in the target cell to provide longer term or enhanced intracellular exposure to the decoy sequence. Plasmids are selected based on  
30 compatibility with the target cell, size and restriction sites, replicative frequency, copy number maintenance, etc. For example, plasmids with relatively short half-lives in the target cell are preferred in situations where it is desirable to maintain therapeutic transcriptional modulation for less than the lifetime of the target cell. Exemplary

plasmids include pUC expression vectors driven by a beta-actin promoter and CMV enhancer, vectors containing elements derived from RSV or SV40 enhancers, etc. The adeno-associated viral vector preferentially integrates in chromosome 19 and may be utilized for longer term expression.

5 The oligonucleotides which are employed may be naturally occurring or other than naturally occurring, where the synthetic nucleotides may be modified in a wide variety of ways, see e.g. Biłńska et al (1990) *Science* 250, 997. Thus, oxygens may be substituted with nitrogen, sulfur or carbon; phosphorus substituted with carbon; deoxyribose substituted with other sugars; or individual bases substituted with an  
10 unnatural base. In each case, any change will be evaluated as to the effect of the modification on the binding of the oligonucleotide to the target transcription factor, as well as any deleterious physiological effects. These modifications have found wide application for "anti-sense" oligonucleotides, so that their safety and retention of binding affinity are well established in the literature. See, for example, Wagner et al.,  
15 *Science* 266, 1510-1513 (1993). The strands may be synthesized in accordance with conventional ways using phosphoramidite synthesis, commercially available automatic synthesizers, and the like.

The administered compositions may comprise individual or mixtures of decoys. Usually the mixture will not exceed 4 different decoys usually not exceed 2. The  
20 decoys are administered to a host in a form permitting cellular internalization of the decoys in an amount sufficient to competitively inhibit the binding of the targeted transcription factor to an endogenous gene. The host is typically a mammal, usually a human. The selected method of administration depends principally upon the target  
25 cell, the nature of the decoy, the host, the size of the decoy. Exemplary methods are described in the examples below; additional methods including transfection with a retrovirus, viral coat protein-liposome mediated transfection, lipofectin etc. are described in Dzau et al., *Trends in Biotech* 11, 205-210 (1993).

Where administered in liposomes, the decoy concentration in the lumen will generally be in the range of about 0.1 uM to 50 uM per decoy, more usually about 1  
30 uM to 10 uM, most usually about 3 uM. For other techniques, usually one will determine the application rate empirically, using conventional techniques to determine desired ranges.

In some situations it may be desirable to provide the decoy source with an agent which targets the target cells, such as an antibody specific for a surface membrane protein on the target cell, a ligand for a receptor on the target cell, etc. For example, for intervention in HIV infection, cells expressing HIV gene products or CD4 may be specifically targeted with gene product or CD4-specific binding compounds. Also, where liposomes are involved, one may wish to include proteins associated with endocytosis, where the proteins bind to a surface membrane protein associated with endocytosis. Thus, one may use capsid proteins or fragments thereof tropic for a particular cell type, antibodies for proteins which undergo internalization in cycling, proteins that target intracellular localization and enhance intracellular half-life.

The application of the subject therapeutics are preferably local, so as to be restricted to a histological site of interest e.g. localized inflammation, neoplasia or infection. Various techniques can be used for providing the subject compositions at the site of interest, such as injection, use of catheters, trocars, projectiles, pluronic gel stents, sustained drug release polymers or other device which provides for internal access, or the like. Where an organ or tissue is accessible because of removal from the patient, such organ or tissue may be bathed in a medium containing the subject compositions, the subject compositions may be painted onto the organ, or may be applied in any convenient way. Alternatively, systemic administration of the decoy using e.g. lipofection, liposomes with tissue targeting (e.g. antibody), etc. may be practiced. Systemic administration is most applicable where the distribution of the targeted transcription factor is primarily limited to targeted cell types, e.g. virus-specific transcription factors limited to infected cells, mutant oncogenic transcription factors limited to transformed cells, etc.

Optimal treatment parameters will vary with the indication, decoy, clinical status, etc., and are generally determined empirically, using the guidance provided herein. Several exemplary indications, routes and vehicles of administration and decoy combinations are disclosed in the following table.

30

INDICATION	ROUTE	VEHICLE	PLASMID/OLIG O
HIV infection	intravenous inj.	gp160 in neutral liposomes	TAR containing oligo
solid tumor	intratumoral inj.	tumor-specific Ab with liposomes	E2F
Inflammatory skin disease and dermatitis	topical	polymer	NFκB, E2F
Hypercholesterol- emia	intravenous inj. portal vein inj.	cationic liposomes asialoglycoprotein receptor targeting with liposomes	sterol responsive element to increase LDL receptors
vein bypass grafts	topical/intraluminal	polymer, liposomes	E2F
glomerulonephritis	intravenous, intrarenal	polymer, liposomes	E2F, NFκB
myocardial infarction	intracoronary	liposomes, polymer	NFκB, E2F, AP-1
organ transplant esp. cardiac/renal	intravascular, ex vivo	liposomes, polymer	NFκB

A wide variety of indications may be treated, either prophylactically or therapeutically with the subject compositions. For example, prophylactic treatment may inhibit mitosis or proliferation or inflammatory reaction prior to a stimulus which would otherwise activate proliferation or inflammatory response, where the extent of proliferation and cellular migration may be undesirable. Similarly, a therapeutic application is provided by a situation where proliferation or the inflammatory response

is about to be initiated or has already been initiated and is to be controlled. The methods and compositions find use, particularly in acute situations, where the number of administrations and time for administration is relatively limited.

Conditions for treatment include such conditions as neoproliferative diseases including inflammatory disease states, where endothelial cells, inflammatory cells, glomerular cells may be involved, restenosis, where vascular smooth muscle cells are involved, myocardial infarction, where heart muscle cells may be involved, glomerular nephritis, where kidney cells are involved, hypersensitivity such as transplant rejection where hematopoietic cells may be involved, cell activation resulting in enhancement of expression of adhesion molecules where leukocytes are recruited, or the like. By administering the decoys to the organ *ex vivo* prior to implantation and/or after implantation, up regulation of the adhesion molecules may be inhibited. Adhesion molecules include homing receptors, addressins, integrins, selectins, and the like.

The following examples are offered by way of illustration and not by way of limitation.

#### EXPERIMENTAL

##### Example 1. Transfection of E2F Decoys into Cultured Cells.

For the nuclear extracts, vascular smooth muscle cells ("VSMCs") were stimulated by serum until confluent. After confluent, the cells were made quiescent by placing in serum free medium for 48 h. After the transfection of decoy oligodeoxynucleotides ("ODN"; 14 bp, (SEQ ID NOS: 01 and 02)) essentially as described in Morishita et al. (1993) *Proc. Natl. Acad. Sci. USA*, 90, 8474-8478, cells were stimulated by 5% serum. After 6 h, RNA was extracted by RNAzol (Tel-Test Inc, Texas) Chomczynski and Sacchi (1987) *Anal. Biochem.* 162, 156-159. Levels of PCNA, *cdc2* and beta-actin mRNAs were measured by RT-PCR (Morishita et al. (1993) supra). The PCNA primer (nucleotides 150-177 of rat PCNA cDNA) and the *cdc2* 5' primer (nucleotides 54-75 of human *cdc2* cDNA) were previously described (Morishita et al. (1993) supra). The primers complementary to the rat beta-actin gene were obtained from Clontech Laboratories Inc. (Palo Alto, CA). Aliquots of RNA were amplified simultaneously by PCR (30 cycles) and compared with a negative control (primers without RNA). Amplification products were electrophoresed through 2% agarose gels stained with ethidium bromide. A gel mobility shift assay was

performed as previously described (Horiuchi et al., *J. Biol. Chem.* 266, 16247-16254 (1991)).

The 14 bp double-strand ODN, (SEQ ID NOS: 01 and 02) effectively abolished the binding of the E2F transcription factor to a specific binding site in serum stimulated VSMCs. See also, Hiebert et al. (1989) PNAS 86, 3594. Transfection of the E2F decoy ODN markedly inhibited the induction of *c-myc*, *cdc2* and PCNA mRNA expression in response to serum stimulation. The E2F decoy ODN had no effect on beta-actin mRNA expression. Furthermore, the control missense E2F element ODN containing two base pair substitutions that eliminate E2F binding failed to inhibit the induction of *c-myc*, *cdc2* and PCNA RNA expression in response to serum stimulation. In association with effective inhibition of cell cycle regulatory gene expression, transfection of the 14 bp E2F decoy also abolished serum-stimulated VSMC proliferation. In contrast, the missense E2F element ODN (SEQ ID NOS:03 and 04) had no effect on mitogenesis induced by serum.

To further confirm the specificity of this response to the E2F decoy, a 30 bp double-stranded ODN (SEQ ID NOS:05 and 06) which contained two 8 base pair E2F cis elements capable of specific binding to E2F was employed (Weintraub et al., *Nature* 358, 259-261 (1992)). In the 30 bp E2F decoy the fifth nucleotide of the 8 bp E2F cis elements was changed from C to G. Despite these differences in flanking sequences and nucleotide composition, both E2F decoys effectively bind E2F and inhibit serum-stimulated vascular smooth muscle cell (VSMC) proliferation. Moreover, a 30 bp missense E2F element ODN (SEQ ID NOS: 07 and 08) with 5 bp substitutions with the 8 bp E2F consensus elements fails to bind E2F and also fails to inhibit serum-stimulated VSMC proliferation. Thus, these *in vitro* studies documented that transfection of the E2F cis element decoy ODN binds the E2F transcription factor, blocked the induction of cell cycle regulatory gene expression and inhibited VSMC proliferation in a sequence specific manner.

#### Example 2. E2F Decoys In Vivo.

Liposomes were prepared as follows: Phosphatidylserine phosphatidylcholine, and cholesterol were mixed in a weight ratio (1:4.8:2) to create a lipid mixture. Lipid was hydrated in a balanced salt solution containing ODN (SEQ ID NOS:01-02) (110 nmol). Purified HVJ(Z) strain was inactivated by UV radiation just before use.

The liposome suspension was mixed with HVJ (Z strain) (20,000 hemagglutinating units), incubated, then free HVJ removed by sucrose density gradient centrifugation. The final concentration of encapsulated DNA was calculated as previously reported (Morishita et al. (1993) supra). This method results in a more rapid cellular uptake and nuclear concentration, leading to a 100-fold higher transfection efficiency of ODN than lipofection or passive uptake methods.

The sequences of the phosphorothioate ODN utilized:

decoys-1 5' -CTAGATTTCCCGCG-3' (SEQ ID NO:01)  
 3' -TAAAGGGCGCCTAG-5' (SEQ ID NO:02)  
 mismatched-1 5' -CTAGATTTCGGAGCG-3' (SEQ ID NO:03)  
 3' -TAAAGCTCGCCTAG-5' (SEQ ID NO:04)

We also examined another set of decoy ODNs containing two binding sites:

decoys-2: 5' -GATCAAAGCGCGAATCAAAGCGCGAATC-3' (SEQ ID NO:05)  
 3' -CTAGTTTTCGCGCTTAGTTTTCGCGCTTAG-5' (SEQ ID NO:06)  
 mis-matched-1 5' -GATCAAAGAACTGAATCAAAGAACTGAATC-3' (SEQ ID NO:07)  
 matched-1 3' -CTAGTTTTCTTGACTTAGTTTTCTTGACTTAG-5' (SEQ ID NO:08)

Rat aortic VSMC (passage 4-10) were studied in a confluent, quiescent state in serum-free media (Morishita et al, *J. Clin. Invest.* 91, 2580-2585 (1993)). The cells were incubated with hemagglutinating virus Japan (HVJ) liposomes (3  $\mu$ M) at 4°C for 5 min and 37°C for 30 min. Three days after transfection in either calf serum (CS) or serum-free media, cell number was determined by Coulter-Counter (Coulter, FL).

### Example 3. Effect of decoy ODN on *in vivo* gene expression.

A 2 French Fogarty catheter was used to induce vascular injury in male Sprague-Dawley rats (400-500 g; Charles River Breeding Laboratories) (Hanke et al., *Circ. Res.* 67, 651-659 (1990)). These rats were anesthetized, and a cannula introduced into the left common carotid via the external carotid artery. After vascular injury of the common carotid, the distal injured segment was transiently isolated by temporary ligatures. The HVJ complex was infused into the segment and incubated for 10 min at room temperature. No adverse neurological or vascular effects were observed in any animal undergoing this procedure.



For RNA analysis, vessels were harvested at 6 h, (c-myc and beta-actin) and one day (cdc2 kinase, PCNA and beta-actin) after transfection. RNA was extracted from mismatched or E2F decoy ODN (3  $\mu$ M) treated injured or untreated intact vessels by RNazol (Tel-Test Inc., TX). RT-PCR was performed as described above.

5 For BrdU staining, BrdU was injected into rats (100 mg/kg subcutaneous and 30 mg/kg intraperitoneal at 18 h prior, and then 30 mg/kg intraperitoneal at 12 h prior to sacrifice (Hanke et al., *supra*). Rats were sacrificed after day four after transfection. The carotid artery was removed after perfusion-fixation with 4% paraformaldehyde, and processed for immunohistochemistry in a standard manner  
10 using anti-BrdU antibodies (Amersham). The proportion of BrdU positive cells was determined by cell counts under light microscopy in a blinded fashion.

Transfection procedures were described above. HYJ-ODN complex (3  $\mu$ M) was administered into the rat carotid injured arteries. At two weeks after transfection, rats were sacrificed and vessels were perfusion-fixed with 4% paraformaldehyde.

15 Three individual sections from the middle of transfected segments were analyzed. In addition, three sections from the middle section of the injured untransfected region were also analyzed. Animals were coded so that the operation and analysis were performed without knowledge of which treatment individual animals received. Intimal and medial areas were measured on a digitizing tablet (Southern Micro Instruments,  
20 GA). Analysis of variance with subsequent Duncan's test was used to determine significant differences in multiple comparisons.  $P < 0.05$  was considered significant.

The scrambled and progesterone responsive element (PRE) decoy sequences utilized as control ODNs are as follows:

25 Scrambled: 5' - TCCAGCTTCGTAGC - 3' (SEQ ID NO:09)  
decoys 3' - GAAGGATCGATCG - 5' (SEQ ID NO:10)

PRE: 5' - GATCCTGTACAGGATGTTCTAGCTACA - 3' (SEQ ID NO:11)  
3' - CTAGGACATGTCCTACAAGATCGATGT - 5' (SEQ ID NO:12)

30 At one day after balloon injury mRNA levels of c-myc, cdc2 and PCNA were elevated in carotid vessels transfected with the control missense E2F element ODN as detected by RT-PCR. However, *in vivo* transfection of the E2F cis element decoy ODN resulted in a marked decrease in c-myc, cdc2 and PCNA mRNA levels to barely detectable levels similar to uninjured vessels. Moreover, the E2F cis element decoys significantly inhibited bromodeoxyuridine (BrdU) incorporation (a marker of DNA  
35 synthesis) within the vessel wall at 4 days post-injury as compared to the missense

E2F ODN material. Furthermore, transfection of the E2F decoy ODN (n=8) resulted in a marked suppression of neointima formation at two weeks after angioplasty compared to vessels treated with the HVJ-liposome complex alone (n=5) or mismatched control decoy ODN-treated vessels (n=8). The selectivity of the decoy ODN effect was further confirmed by the fact that the inhibition of the neointima formation was limited to the area of intraluminal transfection (neointima/medial ratio; transfected lesions =  $0.291 \pm 0.061$  vs. untransfected lesions =  $1.117 \pm 0.138$ ,  $P < 0.01$ ). In contrast, the adjacent injured carotid segments outside the area of decoy transfection exhibited neointimal lesions similar to the mismatched ODN-treated control. Moreover, neither transfection of scrambled decoys (14 bp) nor the progesterone response element decoys (27 bp) (Klein-Hitpass et al., *Cell* 60, 247-257 (1990)) resulted in the inhibition of neointima formation.

The specificity of the inhibitory effect of the decoy ODN against E2F on neointima information is supported by several lines of evidence: (1) the two different E2F decoy ODN bind to the E2F transcription factor in a sequence specific manner and transfection of the E2F decoy ODN completely inhibited VSMC proliferation *in vitro*, whereas the mismatched ODN did not; (2) the *in vitro* and *in vivo* experiments documented that the E2F decoy ODN selectively inhibited the expression of the targeted cell cycle regulatory genes (c-myc, PCNA and cdc2 kinase) transactivated by E2F, but not beta-actin (presumably, other E2F transactivated genes such as c-myb might be inhibited); (3) the E2F decoy ODN specifically reduced a quantitative marker of cell cycle progression *in vivo* (BrdU labeling); (4) the administration of the E2F decoy, but not mismatched, ODN markedly inhibited neointima formation; (5) the prevention of neointima information was limited to the area transfected with the decoy ODN; and (6) neither scrambled nor PRE cis element decoys inhibited the neointima formation.

#### Example 4. Expression of Renin in Cultured Submandibular Gland (SMG) Cell Line

To evaluate the molecular mechanisms which regulate tissue specific renin gene expression, we employed a cell line (SCA-9) derived from a SMG tumor. This line, derived from a two renin gene mouse (Swiss Webster) has been reported to contain renin. Using immunohistochemistry and primer extension analysis, we confirmed by

immunohistochemistry that this cell line expresses renin. Moreover, consistent with the SMG origin of this cell line, only the Ren2 gene is transcribed. To elucidate the mechanism behind the silencing of the Ren1 gene in this cell line, we examined if the negative response element, NRE, binding protein was present in these cells. Nuclear  
5 extracts were prepared from cultured SMG cells and gel mobility shift assay was performed using  $^{32}\text{P}$ -NRE oligonucleotide as a probe. A specific protein: DNA complex was observed which could be specifically competed with unlabeled NRE oligonucleotide.

10 These results show that the pattern of renin expression in this cell line is similar to that seen in the SMG in vivo, validating the use of these cells for studies of the mechanism by which renin expression is regulated.

**Example 5. Use of "Decoy" to Validate the Importance of Cis-Trans Interactions**

15 We examined the feasibility of the "decoy" approach to test whether the NRE:NRE binding protein interaction is responsible for the silencing of the Ren1 gene in the SMG and in the cell line derived from the SMG. This assay is based on the in vivo competition for trans acting factors. The competition is between the endogenous cis elements found in the target gene and exogenously added oligonucleotides  
20 corresponding to that cis sequence. This competition will attenuate the authentic cis-trans interaction, result in the removal of the trans factors from the cis element, with the subsequent modulation of gene expression.

An FITC labeled-double stranded oligonucleotide (33 mer), corresponding to the NRE were introduced into the SMG cell line using the HVJ-liposome technology.  
25 The cells were incubated with HVJ-liposome containing NRE decoy at 4°C for 10 minutes and then at 37°C for 30 minutes. The cells readily took up the oligomers as evidenced by the intense fluorescent signal which was localized in the nucleus (Figure 1C). The cells were harvested 24 hours later, RNA was prepared and subjected to the primer extension analysis to examine the expression of the Ren1 and Ren2 genes.  
30 Consistent with our hypothesis, introduction of the NRE sequences into these cells resulted in the induction of Ren1 gene expression. The expression of the Ren2 gene was not influenced by the administration of the NRE oligonucleotide.

These results show that NRE:NRE binding protein interaction is responsible for the silencing of Ren1 expression in this cell line and demonstrate the feasibility of using the "decoy" approach to examine the cis-trans interactions responsible for the regulation of renin gene expression.

5

Example 6. In Vivo Gene Transfer into the SMG

We transfected 10 ug of our expression vectors into the SMG of a DBA/2J mouse using the HVJ-Liposome-DNA complex as describe above. This was accomplished by injecting directly into multiple regions of the gland with a total volume of 100 ul using a 27 gauge needle. Three days after transfection, the SMG was removed, homogenized and the supernatant assayed for CAT activity. Transfection of pUtk-CAT directed the expression of CAT (Horiuchi et al. (1991) supra). The presence of the Ren1 Xba fragment (containing the CRE/NRE region) decreased CAT expression by approximately 90%. Deletion of the NRE sequence by in vitro mutagenesis resulted in the recovery of CAT expression. These results demonstrate that it is possible to use in vivo gene transfer to examine the expression of genes in the SMG in vivo.

It is evident from the above results, that the subject methods and compositions provide opportunities for preventing injury from acute proliferative diseases or diseases involving expression of proteins, such as proteins associated with cell division, adhesion and/or migration. Thus, the subject methods and compositions can provide safe prophylactic and therapeutic treatments for a number of diseases associated with cellular proliferation and inflammation, resulting in pathogenic conditions.

All publications and patent applications cited in this specification are herein incorporated by reference as if each individual publication or patent application were specifically and individually indicated to be incorporated by reference.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be readily apparent to those of ordinary skill in the art in light of the teachings of this invention that certain changes and modifications may be made thereto without departing from the spirit or scope of the appended claims.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

- (i) APPLICANT: Dzau, Victor J.
- (ii) TITLE OF INVENTION: THERAPEUTIC USE OF CIS-ELEMENT DECOYS IN VIVO

(iii) NUMBER OF SEQUENCES: 2

(iv) CORRESPONDENCE ADDRESS:

- (A) ADDRESSEE: FLEHR, HOHBACH, TEST, ALBRITTON & HERBERT
- (B) STREET: 4 Embarcadero Center, Suite 3400
- (C) CITY: San Francisco
- (D) STATE: CA
- (E) COUNTRY: USA
- (F) ZIP: 94111-4187

(v) COMPUTER READABLE FORM:

- (A) MEDIUM TYPE: Floppy disk
- (B) COMPUTER: IBM PC compatible
- (C) OPERATING SYSTEM: PC-DOS/MS-DOS
- (D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:

- (A) APPLICATION NUMBER: PCT/US94/
- (B) FILING DATE: 28-OCT-1994
- (C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA:

- (A) APPLICATION NUMBER: 08/144,717
- (B) FILING DATE: 28-OCT-1993

(viii) ATTORNEY/AGENT INFORMATION:

- (A) NAME: Rowland, Bertram
- (B) REGISTRATION NUMBER: 20,013
- (C) REFERENCE/DOCKET NUMBER: FP-58506/BIR/DZAU-0001

(ix) TELECOMMUNICATION INFORMATION:

- (A) TELEPHONE: (415) 781-1989
- (B) TELEFAX: (415) 398-3249
- (C) TELEX: 910 277299 FHT UR

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 14 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

CTAGATTTCC CGCG

14

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 14 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

GATCCGCGGG AAAT 14

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 14 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

CTAGATTTCG AGCG 14

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 14 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

GATCCGCTCG AAAT 14

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 30 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

GATCAAAAAGC GCGAATCAAA AGCGCGAATC 30

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 30 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

GATTCGCGCT TTTGATTTCG GCTTTTGATC 30

(2) INFORMATION FOR SEQ ID NO:7:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 30 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: double
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7: UNIDENTIFIED SEQUENCE

GATCAAAGAA CTGAATCAA GAACTGAATC

(2) INFORMATION FOR SEQ ID NO:8:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 30 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: double
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8: UNIDENTIFIED SEQUENCE

GATTCAGTTC TTGATTCAG TTCTTTGATC

(2) INFORMATION FOR SEQ ID NO:9:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 14 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: double
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9: UNIDENTIFIED SEQUENCE

TCCAGCTTCG TAGC 14

(2) INFORMATION FOR SEQ ID NO:10:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 13 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: double
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10: UNIDENTIFIED SEQUENCE

GCTAGCTAGG AAG 13

(2) INFORMATION FOR SEQ ID NO:11:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 27 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: double

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

GATCCTGTAC AGGATGTTCT AGCTACA 27

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 27 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: double

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

TGTAGCTAGA ACATCCTGTA CAGGATC 27



WHAT IS CLAIMED IS:

1. A method of modulating gene transcription *in vivo* within mammalian cells, said method comprising:

5 contacting a mammal with a composition comprising dsDNA having a sequence specific for binding to a transcription factor which modulates the transcription of at least one gene,

whereby said dsDNA is introduced into the nuclei of said cells in an amount sufficient to competitively inhibit the binding of said transcription factor to said gene, whereby the transcription of said gene is modulated.

10

2. A method according to Claim 1, wherein said dsDNA is capable of episomal replication in said cell.

3. A method according to Claim 1, wherein said dsDNA consists essentially of 15 oligonucleotides less than 100 bp in length.

4. A method according to Claim 1, wherein said composition further comprises liposomes and said dsDNA is contained within the lumen of said liposomes.

20 5. A method according to Claim 4, wherein said liposomes comprise lipid and a viral coat protein.

6. A method according to Claim 1, wherein said transcription factor is E2F, AP-1 or NF $\kappa$ B.

25

7. A method according to Claim 1, wherein said cells are vascular smooth muscle cells, tumor cells or endothelial cells..

8. A method for treating a mammalian host to prevent restenosis, said method 30 comprising:

introducing dsDNA into vascular smooth muscle cells at the site of a vascular lesion, said cells capable of resulting in restenosis as a result of neointima formation, in an amount to inhibit said neointima formation,

whereby said dsDNA is characterized by having a sequence specific for binding  
5 to a transcription factor which modulates the transcription of at least one gene, where the transcription product of said gene is necessary for proliferation of said cells.

9. A composition comprising a viral coat protein-liposome containing dsDNA in the lumen of said liposome in a physiologically acceptable medium, wherein said  
10 dsDNA is characterized by having a sequence specific for binding to a transcription factor, wherein said transcription product of said gene is necessary for cell proliferation.

10. A composition according to Claim 12, wherein said viral coat is from the  
15 hemagglutinating virus Japan.

11. A composition according to Claim 12, wherein said transcription factor is E2F, AP-1 or NF $\kappa$ B.

20 12. A composition according to Claim 12, wherein said dsDNA is at a concentration in the range of about 0.1 to 20  $\mu$ M.

INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US94/12339

**A. CLASSIFICATION OF SUBJECT MATTER**  
 IPC(6) : A61K 31/70, 47/44, 48/00; C07H 21/04  
 US CL : 514/44; 536/24.1, 24.5; 424/450  
 According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**  
 Minimum documentation searched (classification system followed by classification symbols)  
 U.S. : 514/44; 536/24.1, 24.5; 424/450

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)  
 APS  
 search terms: transcription factor, gene

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	Mayo Clinic Proceedings, Volume 68, issued 1993, R. S. Schwartz et al., "Coronary Restenosis; Prospects for Solution and New Perspectives from a Porcine Model", pages 54-62, see entire document.	1-12
A	Circulation, Volume 86, No. 4, Supplement I, issued October 1992, H. Hutchinson et al., "Inhibition of Human Vascular Smooth Muscle Proliferation Using c-myc Antisense Oligonucleotides", page I-226, abstract no. 0898.	1-12
A	Science, Volume 260, issued 14 May 1993, K. Ohien, "Molecular Advances in Cardiovascular Biology", pages 916-917, see entire document.	1-12

Further documents are listed in the continuation of Box C.  See patent family annex.

*A* document defining the general state of the art which is not considered to be of particular relevance	*T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
*E* earlier document published on or after the international filing date	*X* document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
*L* document which may throw doubt on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*Y* document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
*O* document referring to an oral disclosure, use, exhibition or other means	*G* document member of the same patent family
*P* document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search: 28 DECEMBER 1994  
 Date of mailing of the international search report: 02 FEB 1995

Name and mailing address of the ISA/US Commissioner of Patents and Trademarks: Box PCT, Washington, D.C. 20231  
 Authorized officer: CHARLES C. P. RORIES, Ph.D. (with signature)

## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US94/12339

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A,P	TIG, Volume 10, No. 6, issued June 1994, E. Rubin et al., "Atherosclerosis in Mice: Getting to the Heart of a Polygenic Disorder", pages 199-204, see entire document.	1-12
A,P	Science, Volume 265, issued 05 August 1994, M. Barinaga, "Gene Therapy for Clogged Arteries Passes Test in Pigs", page 738, see entire document.	1-12
A	Circulation, Volume 86, No. 6, Supplement III, issued December 1992, P. Libby et al., "A Cascade Model for Restenosis, A Special Case of Atherosclerosis Progression", pages III-47 to III 52, see entire document.	1-12
A	Chemical Reviews, Volume 90, No. 4, issued June 1990, E. Uhlmann et al., "Antisense Oligonucleotides: A New Therapeutic Principle", pages 543-584, see entire document.	1-12
A	Biochimica et Biophysica Acta, Volume 1097, issued 1991, P. Hug et al., "Liposomes for the Transformation of Eukaryotic Cells", pages 1-17, see entire document.	4, 5, 9, 10
A	Biotechniques, Volume 6, No. 7, issued 1988, R. Mannino et al., "Liposome Mediated Gene Transfer", pages 682-690, see entire document.	4, 5, 9, 10
Y	The European Heart Journal, Volume 12, Abstract Supplement, issued 1991, M. Ebbecke et al., "Inhibition of Human Arterial Smooth Muscle Proliferation by a c-myc Antisense Oligonucleotide", page 11, abstract no. 177, see entire abstract.	1-12
Y	Gene, Volume 89, issued 1990, H. Wu et al., "Inhibition of in vitro Transcription by Specific Double-Stranded Oligodeoxyribonucleotides", pages 203-209, see entire document.	1-12
Y	Proceedings of the National Academy of Sciences USA, Volume 86, issued May 1989, S. Hiebert et al., "E1A-Dependent Transactivation of the Human MYC promoter is Mediated by the E2F Factor", pages 3594-3598, see entire document.	1-12
Y	Circulation, Volume 86, No. 4, Supplement I, issued October 1992, R. Morishita et al., "In vivo Gene Transfer into Intact Blood Vessels: A Novel and Efficient Method", page I-227, abstract no. 0902, see entire abstract.	1-12

INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US94/12339

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Biochemical and Biophysical Research Communications, Volume 186, No. 1, issued 15 July 1992, N. Tomita et al., "Direct in vivo Gene Introduction into Rat Kidney", pages 129-134, see entire document.	1-12
Y	Molecular and Cellular Biology, Volume 3, No. 11, issued November 1983, M. Ostrowski et al., "Glucocorticoid Regulation of Transcription at an Amplified, Episomal Promoter", pages 2045-2057, see entire document.	2

THE UNIVERSITY OF CHICAGO  
DEPARTMENT OF CHEMISTRY

PHYSICAL CHEMISTRY

1. The rate of reaction between  
nitric oxide and hydrogen  
is given by the equation  
 $k = A e^{-E/RT}$   
where  $k$  is the rate constant,  
 $A$  is the pre-exponential factor,  
 $E$  is the activation energy,  
 $R$  is the gas constant, and  
 $T$  is the absolute temperature.  
The activation energy for this  
reaction is 100 kJ mol<sup>-1</sup>.

2. The rate of reaction between  
nitric oxide and hydrogen  
is given by the equation  
 $k = A e^{-E/RT}$   
where  $k$  is the rate constant,  
 $A$  is the pre-exponential factor,  
 $E$  is the activation energy,  
 $R$  is the gas constant, and  
 $T$  is the absolute temperature.  
The activation energy for this  
reaction is 100 kJ mol<sup>-1</sup>.

3. The rate of reaction between  
nitric oxide and hydrogen  
is given by the equation  
 $k = A e^{-E/RT}$   
where  $k$  is the rate constant,  
 $A$  is the pre-exponential factor,  
 $E$  is the activation energy,  
 $R$  is the gas constant, and  
 $T$  is the absolute temperature.  
The activation energy for this  
reaction is 100 kJ mol<sup>-1</sup>.

4. The rate of reaction between  
nitric oxide and hydrogen  
is given by the equation  
 $k = A e^{-E/RT}$   
where  $k$  is the rate constant,  
 $A$  is the pre-exponential factor,  
 $E$  is the activation energy,  
 $R$  is the gas constant, and  
 $T$  is the absolute temperature.  
The activation energy for this  
reaction is 100 kJ mol<sup>-1</sup>.

5. The rate of reaction between  
nitric oxide and hydrogen  
is given by the equation  
 $k = A e^{-E/RT}$   
where  $k$  is the rate constant,  
 $A$  is the pre-exponential factor,  
 $E$  is the activation energy,  
 $R$  is the gas constant, and  
 $T$  is the absolute temperature.  
The activation energy for this  
reaction is 100 kJ mol<sup>-1</sup>.

6. The rate of reaction between  
nitric oxide and hydrogen  
is given by the equation  
 $k = A e^{-E/RT}$   
where  $k$  is the rate constant,  
 $A$  is the pre-exponential factor,  
 $E$  is the activation energy,  
 $R$  is the gas constant, and  
 $T$  is the absolute temperature.  
The activation energy for this  
reaction is 100 kJ mol<sup>-1</sup>.

7. The rate of reaction between  
nitric oxide and hydrogen  
is given by the equation  
 $k = A e^{-E/RT}$   
where  $k$  is the rate constant,  
 $A$  is the pre-exponential factor,  
 $E$  is the activation energy,  
 $R$  is the gas constant, and  
 $T$  is the absolute temperature.  
The activation energy for this  
reaction is 100 kJ mol<sup>-1</sup>.

8. The rate of reaction between  
nitric oxide and hydrogen  
is given by the equation  
 $k = A e^{-E/RT}$   
where  $k$  is the rate constant,  
 $A$  is the pre-exponential factor,  
 $E$  is the activation energy,  
 $R$  is the gas constant, and  
 $T$  is the absolute temperature.  
The activation energy for this  
reaction is 100 kJ mol<sup>-1</sup>.

9. The rate of reaction between  
nitric oxide and hydrogen  
is given by the equation  
 $k = A e^{-E/RT}$   
where  $k$  is the rate constant,  
 $A$  is the pre-exponential factor,  
 $E$  is the activation energy,  
 $R$  is the gas constant, and  
 $T$  is the absolute temperature.  
The activation energy for this  
reaction is 100 kJ mol<sup>-1</sup>.